Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho

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Semaphorins and their receptors, plexins, are widely expressed in embryonic and adult tissues. In general, their functions are poorly characterized, but in neurons they provide essential attractive and repulsive cues that are necessary for axon guidance [1–3]. The Rho family GTPases Rho, Rac, and Cdc42 control signal transduction pathways that link plasma membrane receptors to the actin cytoskeleton and thus regulate many actin-driven processes, including cell migration and axon guidance [4–7]. Using yeast two-hybrid screening and in vitro interaction assays, we show that Rac in its active, GTP bound state interacts directly with the cytoplasmic domain of mammalian and *Drosophila* B plexins. Plexin-B1 clustering in fibroblasts does not cause the formation of lamellipodia, which suggests that Rac is not activated. Instead, it results in the assembly of actin:myosin filaments and cell contraction, which indicates Rho activation. Surprisingly, these cytoskeletal changes are both Rac and Rho dependent. Clustering of a mutant plexin, lacking the Rac binding region, induced similar cytoskeletal changes, and this finding indicates that the physical interaction of plexin-B1 with Rac is not required for Rho activation. Our findings that plexin-B signaling to the cytoskeleton is both Rac and Rho dependent form a starting point for unraveling the mechanism by which semaphorins and plexins control axon guidance and cell migration.

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Results and discussion

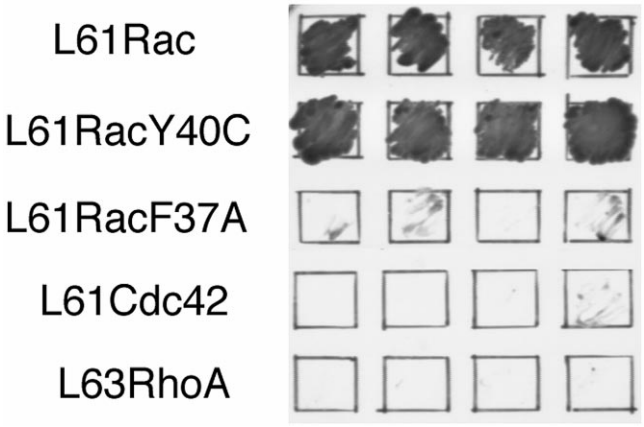
To identify proteins that interact with Rac and are possibly involved in the regulation of the actin cytoskeleton, we performed a yeast two-hybrid screen with a human-brain cDNA library and a mutant version of Rac (L61RacY40C) as a bait. L61RacY40C is constitutively active and induces lamellipodia, but because of the changed tyrosine residue in the effector site, it does not interact with at least three of the known Rac targets, $p65^{PAK}$, POSH, and MLK [8, 9]. Sequence analysis of two of the plasmids rescued from yeast clones shows complete amino acid sequence identity to the C-terminal 412 amino acids (residues 1724–2135) of the cytoplasmic domain of human plexin-B1, a transmembrane receptor for semaphorins [1, 2, 10]. Figure 1 shows that plexin-B1 interacts with L61Rac and the original bait, L61RacY40C, but that it does not interact with Cdc42 or Rho in yeast. It does not interact with L61RacF37A, an effector site mutant of Rac that is unable to induce lamellipodia or to activate Rho [8]. All GTPase bait constructs interact with a fragment of p50RhoGAP (results not shown).

To test whether the interaction of plexin-B1 with Rac is GTP dependent, we used an in vitro dot blot assay [9]. Recombinant plexin-B1 protein (amino acids 1724–2135) was spotted onto nitrocellulose filters and probed with the GTP and GDP bound forms of wild-type Rac1. As seen in Figure 2a, plexin-B1 interacts with GTP bound Rac but not with GDP bound Rac, similarly to PAK, a well-characterized Rac target.

Many previously identified Rac targets contain a distinctive Rac binding site, the CRIB motif, but sequence analysis did not reveal any obvious CRIB-like sequence in plexin-B1 [11]. To identify the region of plexin-B1 that contains the Rac interaction site, we expressed a series of truncations as GST fusion proteins in *E. coli* and used them in a dot blot assay. Rac interacts with a region encompassing 180 residues (amino acids 1724–1903) of the receptor (results not shown).

Plexin-B1 is a member of a large family of transmembrane proteins, and based on sequence alignments, four classes of plexins (A, B, C, and D) have been described [2, 3]. To test whether Rac could interact directly with other members of the family, we obtained cDNAs for human plexin-A2 (kiaa0463), plexin-B2 (kiaa0315), and plexin-D1 (kiaa0620) and cloned a region corresponding to amino acids 1724–1903 of plexin-B1 into the pGEX vector. GST

Figure 1



The interaction of Rho, Rac, and Cdc42 with plexin-B1. Yeast strains containing the Rho GTPases in the integrated bait vector pYTH9 were transformed with the pACTII prey vector containing the plexin-B1 C terminus (amino acids 1724–2135) and plated on minus Leu, minus Trp plates as described previously [22]. Colonies of equal size were replated in the presence of 3-aminotriazole on minus Leu, minus Trp, minus His plates and allowed to grow for 3 days at 30°C.

fusion proteins were analyzed in the dot blot assay, but under these conditions only plexin-B1 was found to interact (data not shown). As shown in Figure 2b,c, in an overlay assay L61Rac interacted strongly with plexin-B1 and also showed some interaction with plexin-B2 but not with plexin-A2 or plexin-D1.

In *Drosophila*, two plexins have been identified, *Drosophila* plexin-A and *Drosophila* plexin-B [3]. As shown in Figure 2d, recombinant *Drosophila* plexin-B protein (C-terminal 435 amino acids, similar to plexin-B1 two-hybrid clone) interacts strongly with in vitro translated *Drosophila* L61Rac1 and weakly with wild-type *Drosophila* Rac1 in a pull-down experiment. *Drosophila* plexin-A does not interact with *Drosophila* Rac1 under the same conditions (data not shown). A *Drosophila* plexin-B fragment (Figure 2e, Bdeletion) corresponding to amino acids 1724–1903 of human plexin-B1 interacted similarly with *Drosophila* Rac1, as did a shorter, 149 amino acid region (Figure 2e, Bdeletion 3). Partial binding was observed with a 54 amino acid domain (Figure 2e, Bdeletion 6).

Maestrini et al. identified two blocks of sequence similarity, of approximately 320 and 150 amino acids each, in plexin cytoplasmic domains. These two blocks of sequence similarity were separated by a variable linker [1]. This linker region is most divergent between the plexin subfamilies. The minimal Rac binding region in *Drosophila* plexin-B consists of the last 149 amino acids of the first conserved block but does not contain the linker region. Alignment of this 149 amino acid region of *Drosophila* plexin-B with other human plexins (Figure 2f) reveals a sequence highly conserved among all plexin subfamilies.

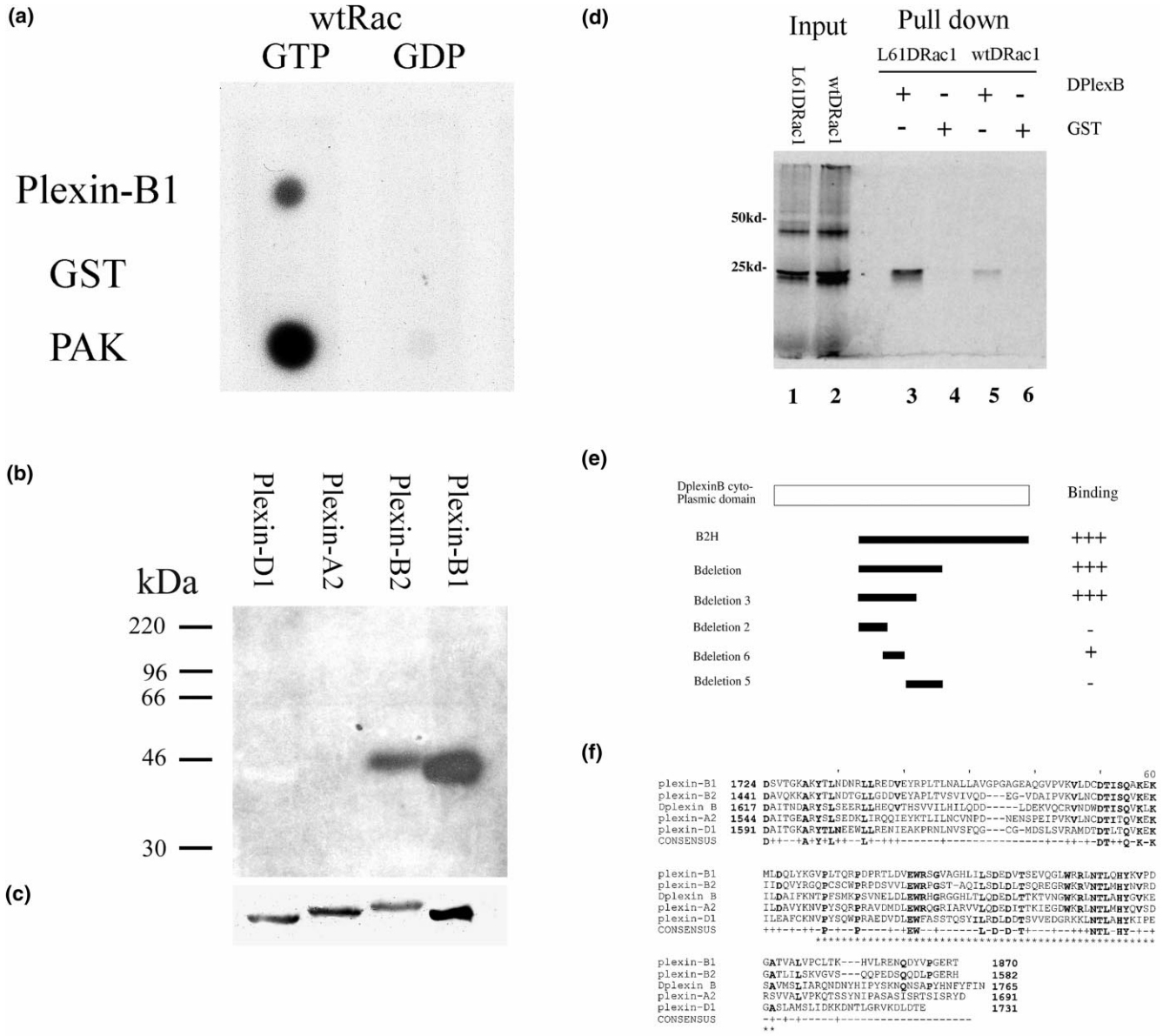
We have shown that the cytoplasmic domains of mammalian plexin-B1 and plexin-B2 and *Drosophila* plexin-B interact with Rac in a GTP-dependent manner. After submission of this paper, a manuscript reporting identical results was published [12]. The human plexin-B subfamily currently includes one additional member, plexin-B3 (kiaa1206), that we have not tested. Northern blot analysis reveals that plexin-B1 is widely expressed in fetal and adult tissues such as brain, lung, liver, and kidney and in neural, hematopoietic, and endothelial cells, but its biological role is unclear [1, 2].

To test whether plexin-B1 can induce changes to the actin cytoskeleton, we subcloned full-length plexin-B1 [10] into the mammalian expression vector pRK5 with a FLAG tag at its C terminus, and the plasmid DNA was microinjected into confluent, serum-starved Swiss 3T3 fibroblasts. Plexin-B1 was strongly expressed after 4–8 hr and localized at the plasma membrane, but no detectable effect on the actin cytoskeleton was observed.

Like other semaphorins, Sema4D (CD100), a transmembrane ligand for plexin-B1, forms multimers and is thought to activate plexin receptors through clustering [2, 13, 14]. To test whether clustering of plexin-B1 can affect actin assembly, we constructed a chimeric molecule (CD2/plexin-B1) in which the cytoplasmic domain of plexin-B1 was fused to the transmembrane and extracellular domains of CD2 [15]. CD2/plexin-B1 DNA was microinjected into serum-starved Swiss 3T3 cells, and 5 hr later surface expression could be detected (data not shown). The chimeric protein was crosslinked by the addition of a monoclonal antibody to CD2, followed by a 15 min incubation with a secondary antibody. As can be seen in Figure 3a,b, crosslinking CD2/plexin-B1 resulted in the formation of stress fibers accompanied by some cell contraction, a phenotype found upon Rho activation [6]. No membrane ruffling could be detected by time lapse video microscopy after crosslinking (data not shown), and no lamellipodial actin could be detected by immunofluorescence (Figure 3b). Crosslinking of a tailless CD2 construct, consisting of the extracellular and transmembrane domains only, did not affect the actin cytoskeleton (Figure 3c,d). We conclude that the crosslinking of plexin-B1 activates Rho, not Rac.

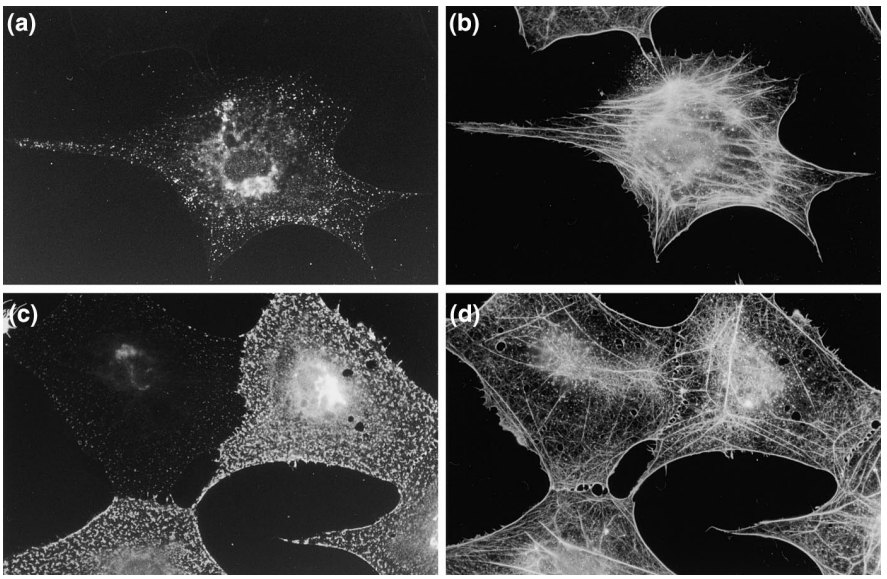
To determine whether the changes in the actin cytoskeleton were mediated by Rho GTPases, we coexpressed CD2/plexin-B1 with inhibitors of Rho, Rac, or Cdc42. As can be seen in Figure 4b, the expression of dominant-negative (N17) Rac completely blocked the formation of the actin stress fibers and cell contraction induced by receptor crosslinking. The coinjection of the Rho inhibitor

Figure 2



(a) Plexin-B1 interacts with Rac in a GTP-dependent manner. Two micrograms of GST–plexin-B1 (amino acids 1724–2135), GST, and GST-p65^{PAK} were spotted on strips of nitrocellulose and then probed with wild-type Rac bound to [α^{32}P]GTP or [α^{32}P]GDP, as described previously [9]. **(b)** Three micrograms of GST–plexin-D1 (amino acids 1591–1754), GST–plexin-A2 (amino acids 1544–1740), GST–plexin-B2 (amino acids 1441–1611), or GST–plexin-B1 (amino acids 1724–1903) were separated on an SDS-PAGE gel, blotted onto nitrocellulose, and tested for interaction with [γ^{32}P]GTP L61Rac. **(c)** Loading of the GST fusion proteins as visualized by Indian ink protein stain of the blot in (b). **(d)** Pull-down assay of in vitro translated, 35S-labeled wild type and *Drosophila* L61Rac1, for which we used 2 μg *Drosophila* plexin-B GST fusion protein (the B2H clone; 435 amino acids; residues 1617–2051) or 2 μg GST as a control. Bound proteins were eluted from the beads with SDS sample buffer and subjected to SDS-PAGE and autoradiography. **(e)** Identification of a 149 amino acid fragment of the cytoplasmic domain of *Drosophila* plexin-B that is sufficient for binding to *Drosophila* L61Rac1. GST fusion proteins (2 μg) of *Drosophila* plexin Bdeletions were used to pull down in vitro translated *Drosophila* L61Rac1. From top to bottom, the locations of the Bdeletions are as follows: B2H fragment (435 amino acids; residues 1617–2051); Bdeletion (211 amino acids; residues 1617–1827); Bdeletion 3 (149 amino acids; residues 1617–1765); Bdeletion 2 (72 amino acids; residues 1617–1688); Bdeletion 6 (54 amino acids; residues 1680–1733); and Bdeletion 5 (90 amino acids; residues 1738–1827). **(f)** Clustal W alignments of the region of 149 amino acids of *Drosophila* plexin-B that retains full binding activity to the homologous regions of plexin-B1 (kiaa0407), plexin-B2 (kiaa0315), plexin-A2 (kiaa0463), and plexin-D1 (kiaa0620). Amino acids that are identical in *Drosophila* plexin-B, plexin-B1, and plexin-B2 are shown in bold. Conserved amino acids are indicated by a plus sign. An asterisk indicates the 54 amino acid region in *Drosophila* plexin-B that was found to contain partial Rac binding activity. Recombinant plexin proteins were generated by PCR with pBluescript cDNA vectors as templates and were produced as fusion proteins with glutathione-S-transferase (GST) in *Escherichia coli* with the pGEX expression vector, as described previously [9]. Recombinant Rac and C3 transferase were expressed as GST fusion proteins and were cleaved by thrombin; active protein concentrations were determined as previously described [9]. The protein concentration was assayed by the Bradford method.

Figure 3



The effects of crosslinking a chimeric CD2/plexin-B1 on the actin cytoskeleton. Quiescent Swiss 3T3 cells were replated on glass coverslips coated with fibronectin, left for 16 hr in serum-free DMEM as described previously [8], and then the nuclei of 100 cells were injected with an expression vector containing **(a,b)** CD2/plexin-B1 or **(c,d)** CD2/tailless at a concentration of 0.1 mg/ml. After 5 hr incubation, we crosslinked the membrane-localized CD2 proteins by using the OX34 mouse anti-rat CD2 mAb at 10 μg/ml for 20 min at 4°C, followed by donkey anti-mouse IgG at 15 μg/ml for 15 min at 37°C. Cells were fixed and stained as described [8]. (a,c) CD2 expression and (b,d) F actin organization are shown. Upon crosslinking, 90% of cells expressing CD2/plexin-B1 (n = 209) showed actin stress fibers, as compared with 22% of the cells expressing CD2/tailless (n = 205). The scale bar represents 10 μm. OX34 followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs) was used for the detection of CD2 expression. Cells were stained for filamentous actin with rhodamine-conjugated phalloidin (at 0.1 μg/ml, Sigma) in PBS. Coverslips were mounted in moviol containing p-phenylenediamine as an antifade agent and were examined on a Zeiss axiophot microscope with 40× and 63× oil immersion lenses. Fluorescence images were recorded on Kodak T-MAX 400 ASA film.

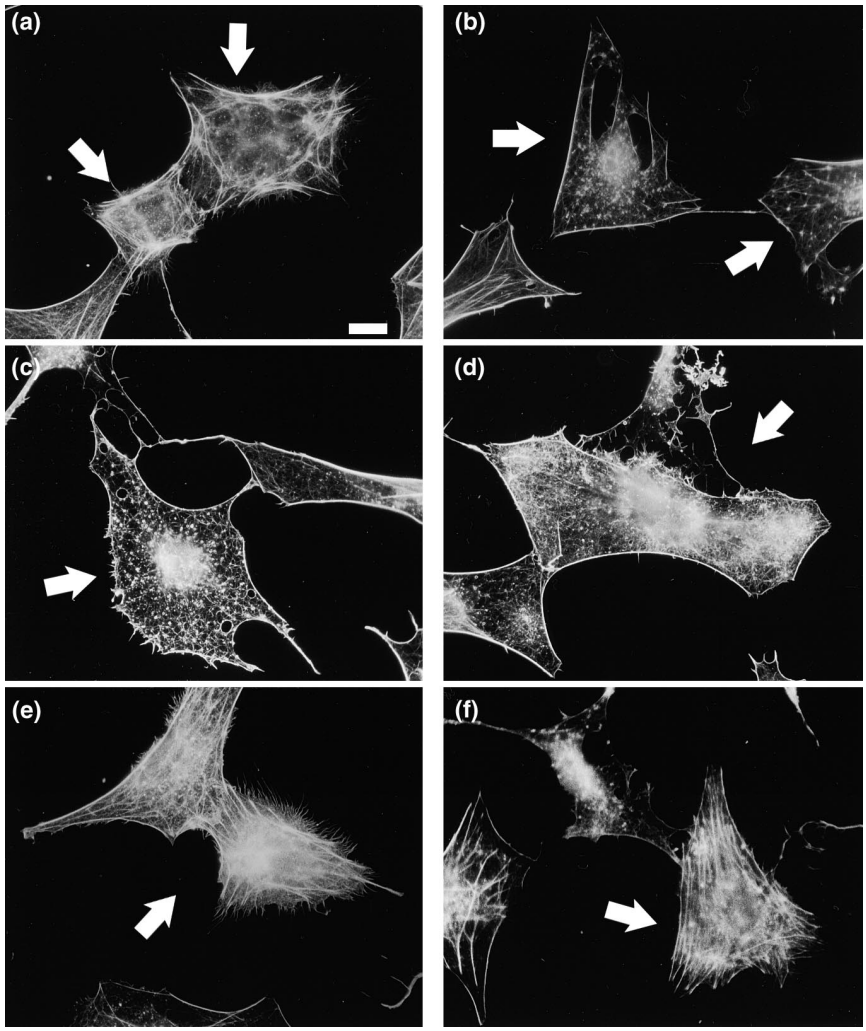
C3 transferase (Figure 4c) or the addition of the Rho-kinase inhibitor Y-27632 (Figure 4d) also completely blocked the cytoskeletal effects. The inhibition of Cdc42, by dominant-negative (N17) Cdc42 (Figure 4e) or by the Cdc42 binding domain of the Wiscott-Aldrich syndrome protein (WASP) (Figure 4f), had no effect on the induced cytoskeletal changes.

To assess the role of the plexin-B1 Rac binding site, we generated a mutant CD2/plexin chimera in which amino acids 1724–1885 (Figure 2f) had been deleted. Upon the microinjection into quiescent Swiss 3T3 cells and subsequent crosslinking of this chimera, we observed the formation of contractile actin bundles, an identical effect to that seen with the chimera that had a complete plexin cytoplasmic domain (results not shown). Formation of these actin bundles by the plexin-B1 chimera lacking a Rac binding site was also blocked by the Rho inhibitor C3 transferase or by dominant-negative Rac (results not shown). It appears that under these conditions, Rac binding to the plexin-B1 cytoplasmic domain is not required for Rho activation. The role of the Rac binding domain is, therefore, unclear. One possibility is that Rac is involved in ligand-induced receptor clustering. Another, perhaps more likely, possibility is, however, that the Rac interaction induces some other change to the receptor, such as endocytosis or redistribution. Further experiments will be required to address this.

Since the cytoplasmic domain of plexins contains no dbl homology (DH) domain, the hallmark of Rho guanine nucleotide exchange factors (RhoGEFs), we think that Rho activation might be mediated by a RhoGEF that is recruited upon plexin clustering [16]. Rac-dependent activation of Rho has been reported previously for other receptors, such as those for PDGF and insulin [7]. However, unlike tyrosine kinase receptors, which are upstream activators of Rac and which lead to lamellipodia formation, plexin-B receptors do not appear to activate Rac but instead interact with activated Rac. One possible explanation for the inhibitory effect of dominant-negative Rac on Rho activation is that the RhoGEF involved is a dual-function Rac/RhoGEF like Trio and that this GEF is inhibited by both dominant-negative Rac and Rho [17]. Interestingly, Trio has been shown to regulate axon guidance [17].

Three papers have reported that growth cone collapse induced by Sema3A acting through the plexin-A1/neuropilin receptors is Rac dependent [18–20]. This is an unexpected result given the important role for Rac in the regulation of neurite extension in vitro and the promotion of growth cone migration in vivo [5, 17, 21]. Although we were unable to detect a direct interaction between Rac and plexin-A receptors, our results could provide the basis for an explanation of why Sema3A/collapsin-1–induced effects are Rac dependent, namely that plexin-mediated Rho activation is Rac dependent. In agreement with this, preliminary observations in *Drosophila* have revealed a genetic interaction between Rac and both plexin-B and plexin-A (H. H. and C. S. G., unpublished data), and it is possible, therefore, that all plexins might be functionally dependent on Rac in vivo.

Figure 4



Both Rac and Rho are necessary for stress fiber formation induced by crosslinked CD2/plexin-B1. F actin distribution is shown in Swiss 3T3 cells microinjected with CD2/plexin-B1 either **(a)** alone, **(b)** coinjected with pRK5-N17Rac, **(c)** coinjected with C3 transferase at a final concentration of 30 μg/ml, **(d)** pretreated for 3 hr and continuously incubated with 5 μM Y-27632 Rho-kinase inhibitor, **(e)** coinjected with pRK5-N17Cdc42, or **(f)** coinjected with the pRK5-Cdc42 binding domain (amino acids 201–321) of WASP. Microinjected cells are marked with an arrow. The scale bar represents 10 μm. Biotin-conjugated mAb 9E10, followed by AMCA-S-conjugated streptavidin (Molecular Probes), was used for the detection of Myc-tagged constructs. In the case in which costaining for CD2 and Myc was required, first the staining for CD2 was carried out, and subsequently any free anti-mouse IgG sites were blocked with mouse IgG at 100 μg/ml; then incubation with the biotin-conjugated 9E10 mAb was performed.

We propose a mechanism for plexin-B signaling to the actin cytoskeleton. In this mechanism, clustering of B plexins induces a Rac-dependent activation of Rho. These results provide a framework for the further exploration of the complex mechanisms by which plexins affect the actin cytoskeleton in different cell types, including neurons.

Supplementary material

A Supplementary materials and methods section is available with the electronic version of this article at http://current-biology.com/supmatin.htm.

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